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We have designed and synthesized a novel compound (11β) that efficiently triggers apoptosis in prostate cancer cells such as LNCaP. This bifunctional compound was designed to form DNA adducts that are camouflaged by the androgen receptor making them less readily repaired in AR+ prostate cancer cells. The aims of our studies are to investigate the mechanisms by which 11β is able to trigger apoptosis in target cells. One approach we are taking is to prepare structural analogs of 11β that have increased or decreased abilities to cause apoptosis in LNCaP cells. Methods have been developed that will permit us to determine the fates of 11β-DNA adducts in treated cells as well as in target and nontarget tissues in xenograft mouse models of human prostate cancer. Another objective is to identify the signaling events that lead from DNA adducts to activation of the apoptotic program. Finally we have obtained encouraging results from animal experiments that indicate that molecules such as 11β may have clinical potential for the treatment of human tumors.

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## INTRODUCTION

The objective of our research is to develop more effective therapeutics for the treatment of prostate cancers. One novel bifunctional compound (**11β**) that we have prepared rapidly induces apoptosis in several prostate cancer cell lines in vitro. The **11β** compound contains a chemically reactive nitrogen mustard linked to a steroid moiety that binds with high affinity to the androgen and progesterone receptor proteins. This compound was designed to create DNA adducts that form tight complexes with these steroid receptors that make the adducts difficult to repair in prostate cancer cells. Preliminary studies of **11β** in cell culture indicated that its effects on prostate cancer cells were different from those of other alkylating agents used in chemotherapy. The apoptotic responses of prostate cancer cells suggested that the **11β** compound might be a useful agent for chemotherapy. The Specific Aims of our research are to understand the fate of **11β**-DNA adducts in treated cells and investigate the mechanisms that lead to apoptosis. We also proposed experiments to assess the antitumor potential of **11β** in animal models of human prostate cancer.

## BODY

**Task 1**: *Chemical synthesis of monochloro and 17α-methyl analogs of our lead compound, 11β-(17αOH-estradien-4(5),9(10)-3-one)-C6NC2-mustard (11β), and assessment of their toxicity in prostate cancer cells in vitro.*

The chemical synthesis of the monochloro and 17α-methyl analogs that were the initial focus of this task has been completed as described in the FY 2004 Progress Report.

During the last year we studied the responses of LNCaP prostate cancer cells to both the monochloro and 17α-methyl analogs. The outcomes of our structure-activity studies have identified several features of the active 11β compound responsible for its ability to induce apoptosis in resistant prostate cancer cell lines. We initially focused our investigations on structure-activity relationships of the reactive N,N-*bis*-(2-chloroethyl)-aniline. Because it has two 2-chloroethyl groups 11β can form monoadducts, intra- and inter-strand crosslinks. Substitution of one or both chlorine atoms by methoxyl groups produced 11β analogs that were either unreactive (i.e., both chlorines replaced) or had a single reactive 2-chloroethyl group (i.e. one chlorine replaced) that could only form monoadducts. Investigation of the cytotoxic effects of these analogs revealed that the apoptotic response depends absolutely on the presence of <u>both</u> of the 2-chloroethyl groups in 11β – implying that apoptosis requires the formation of intra- or inter-strand crosslinks. It is clear, however, that features other than the N,N-*bis*-(2-chloroethyl)-aniline moiety in 11β are essential, because chlorambucil and melphalan, both of which have bifunctional aniline mustards, fail to induce apoptosis in LNCaP cells. Our studies led to the conclusion that the ability to form bifunctional adducts that interact with a steroid receptor is key to the ability to induce apoptosis.

As we previously reported, the affinity of 17α-methyl-11β for the androgen receptor was identical to that of the original 17α-H-11β compound. Both molecules bound to the AR

with affinities of approximately 25% that of dihydrotestosterone. We speculated that the 17-α methyl 11β analog would be more potent than the original 17α-H 11β because the methyl group would be expected to block the oxidative metabolism of the 17-OH position. Oxidation of the 17-OH to a ketone would significantly reduce affinity for the AR. Contrary to our expectations, there was the presence of the methyl group had no effect on the biological activities of 11β. Since preparation of the 17-α methyl 11β compound requires several additional steps we decided not to investigate it further at this time as the original compound can be made more efficiently for *in vivo* studies.

**Task 2**: *Determine the fate of **11β**-DNA adducts in prostate cancer cells.*

As described in the FY 2004 Progress Report, began examination of the kinetics of formation and repair of 11β-DNA adducts in prostate cancer cells using electrospray ionization mass spectometry (EIMS). The EIMS technique did not prove sensitive enough to accurately quantify DNA adducts in cell culture experiments. We then developed a highly sensitive method for their analysis that is based on the technique of Accelerator Mass Spectrometry (AMS). These experiments have been exceptionally successful in the detection and quantification of 11β-DNA adducts in DNA isolated from cells in culture as well as from xenograft tumors in mice. A radiolabeled 11β analog was prepared by modification of its chemical synthesis to allow incorporation of one $^{14}C$ atom into the linker connecting the steroid and aniline mustard moieties. The sensitivity of the AMS technique – which is up to 7 orders of magnitude greater than conventional liquid scintillation counting – has permitted detection of as little as a few hundred DNA adducts in [$^{14}C$]11β-treated cells. In our initial studies with LNCaP cells *in vitro* we examined the relationships between both 11β concentration and exposure time with the level of DNA adducts. Results based on further refinements of analytical protocols are shown in Figure 1. Increasing levels of 11β-DNA adducts were detected by AMS analysis of DNA isolated from LNCaP cells treated for various times with 10 μM [$^{14}C$]-β11 *in vitro*. DNA isolated from approximately $1x10^6$ cells was used for each analysis. We have also performed an initial experiment in an animal model. Mice with LNCaP tumors growing as xenografts were treated with 50 mg/kg [$^{14}C$]-11β (1 μCi $^{14}C$; specific activity 0.48 mCi/mmol) and DNA isolated from liver and tumor tissues after 4 h. Based on the results of this experiment we are able to detect ~600 11β-DNA adducts per cell in tumor tissue.

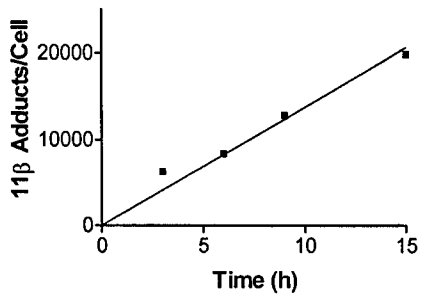


**Figure 1**. Accelerator mass spectrometry analysis of [$^{14}C$]11β-DNA adducts in LNCaP cells treated with 10 μM [$^{14}C$]11β.

The results of these studies will permit us to: (1) examine the rate of repair of DNA adducts in receptor-positive and –negative cells to test the hypothesis that DNA adducts

concealed by the androgen receptor are less well repaired; and (2) define the quantitative relationship between adduct level and cytotoxic effects to facilitate optimization of dosing protocols in animal studies.

**Task 3**: *Determine which apoptotic pathways are activated in prostate cancer cells responding to 11β.*

One aim of these experiments was to uncover the reasons for the ability of 11β to overcome the resistance of LNCaP and other prostate cancer cells to other alkylating drugs. We began to investigate the pathways and mechanisms that lead to apoptosis by identification of the initiator and effector caspases that are activated by 11β. Initial experiments found that the pan-caspase inhibitor ZVD.fmk prevented cleavage of PARP confirming that caspase activation was, in fact, triggered in LNCaP cells by 11β.

The results of our structure-activity studies with and monochloro analog indicated that a specific type of DNA adduct was required for activation of apoptosis. These findings have refocused our investigations on biochemical changes that are more directly related to DNA damage. Our present goal was to investigate the immediate consequences of 11β-DNA adducts on DNA repair and cell cycle checkpoints – at the beginning of the apoptotic pathways. Thus, we have focused on the immediate consequences of the crosslinks formed by 11β in LNCaP cells. We have found evidence of activation of the p53 pathway which is consistent with other observations including increased levels of p21. Several sites on p53 can be phosphorylated in response to DNA damage. Phosphorylation at ser15 occurs as soon as 4-8hr after addition of 11β-dichloro. This phosphorylation is absent when cells are treated with the dimethoxy derivative, indicating that DNA damage is responsible.

We have also looked for phosphorylation of the cell cycle check point kinases Chk-1 and Chk-2. Following addition of 11β-dichloro to cultures of LNCaP cells, evidence of Chk-1 phosphorylation was seen only after 16hr. Similarly, Chk-2 (thr 68) phosphorylation, was seen after 24hr. The timing of Chk-1 and Chk-2 phosphorylation occurs well after other biochemical changes that commit cells to apoptosis suggesting that the inhibition of these responses by 11β may be important.

During the next year we shall continue to investigate the responses of p53, Chk-1 and Chk-2 by comparing those of 11β-dichloro with other compounds. We shall conduct experiments to determine whether the 11β-monochloro analog or chlorambucil can activate the p53 or Chk pathways LNCaP cells. Discovery of changes in either pathway that are specific to 11β will direct further studies toward identification of the biochemical basis for this compound's effectiveness.

**Task 4**: *Assess the efficacy of 11β in an animal xenograft model of human prostate cancer.*

As reported in our FY 2004 Progress Report, the 11β compound is very effective in preventing the growth of LNCaP cell in mouse xenografts. It is especially encouraging that the dose of 11β that was effective in preventing tumor growth did not show significant toxic effects in the animals. During the past year we have sought to optimize the treatment protocol and method of delivery of 11β to increase the antitumor response.

In our inintial investigations of the antitumor activity of 11β we have administered the compound dissolved in a vehicle containing Cremophor EL, a polyoxyethylated castor oil. The availability of radiolabeled compound enabled us to determine its plasma levels and tissue distribution following i.p. injection. These investigations revealed that 11β was rapidly absorbed and reached pharmacological concentrations in the blood shortly after administration. The maximum concentration of 11β in the blood (Cmax) occurred within 30 min and then declined with a half life of approximately 1.3 h. Some of our pharmacokinetic and biodistribution data are shown in Figure 2. They indicate that delivery of 11β into the peripheral circulation and tumor tissue was likely limited by its removal from the circulation by the liver and subsequent transport into the intestine/feces. These results pointed to the need for additional investigation of formulation, delivery and dosing schedules that could significantly enhance tumor response.

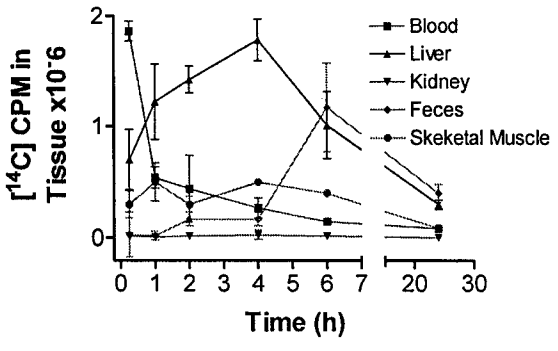


**Figure 2.** Distribution of [$^{14}$C]-11β in tissues and blood after i.p. injection.

We have approached this problem by first seeking an improved vehicle in which to administer the compound. One reason for this was that Cremophor EL in our current vehicle poses potential problems of clinically significant side effects including acute hypersensitivity reactions and peripheral neurotoxicity. Another reason is that we believe that a more optimal pharmacokinetic profile for 11β can be obtained by formulating the compound in a manner that slows its uptake by the liver. Increasing the plasma half life of 11β would allow us to decrease the total dose required to reach effective concentrations in target tissues and perhaps lower its potential to produce adverse side effects. Recent formulation studies on 11β in work with the Sasishakran lab here at MIT revealed that the physio-chemical properties of 11β make it ideal for incorporation into long-circulating PEG-coated liposomes that can evade uptake by the reticulo-endothelial (RE) system. The liposome-based formulations of other hydrophobic anticancer drugs such as doxorubicin and paclitaxel are reported to be effective and have fewer adverse side effects formulations in which these drugs are administered in Cremophor EL or other

vehicles. Selective extravascular accumulation of liposome formulations in tumor xenografts has been reported to result from increased microvascular permeability that is characteristic of vessels in the hypoxic environments of solid tumors. Our initial results are shown in Figure 3 indicate persistence in blood over a 10 h period of the liposome-encapsulated 11β when injected into the tail vein.

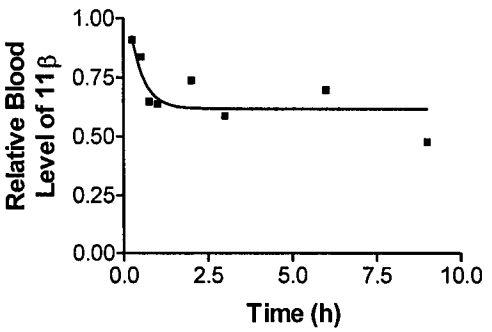


**Figure 3.** Relative levels of [$^{14}$C]-11β in blood after injection of 11β-liposomes.

During the next year we shall continue to investigate the pharmacokinetics of 11β in liposomes and assess its delivery to tumor and normal tissues in a xenograft mouse model of prostate cancer using [$^{14}$C]-11β. Traditional liquid scintillation counting methods will be used to determine the amount of 11β in each tissue, while the much more sensitive AMS analysis will be used to quantify 11β-DNA adducts that are formed in target and nontarget tissues. The measurement of DNA adducts will provide a direct assessment of the amount of biologically active compound reaching the desired molecular target within the tumor and allow us to investigate dosing parameters (e.g., amount, frequency and route of administration). We will also seek to optimize the dose and schedule of administration to enhance the antitumor responses.

## KEY RESEARCH ACCOMPLISHMENTS

- Evaluation of toxicity of the monochloro-11β and 17α-methyl-11β compounds. The absence of apoptotic responses in LNCaP cells treated with monochloro-11β indicated bis-DNA adducts are required for apoptosis.

- Optimization of a sensitive method for the kinetic analysis of DNA adducts formed in cells treated *in vitro* with [$^{14}$C]-11β and application of the method to the identification and quantification 11β-DNA adducts in xenograft tumors. These results have led to experiments that will define the dose relationship between cytotoxic responses *in vitro* and therapeutic responses in animal models.

- Discovery that the p53 pathway was activated in LNCaP cells by 11β-dichloro while the Chk-1 and Chk-2 responses are absent. These results have pointed to identify the molecular basis of the apoptotic responses of LNCaP cells to 11β-dichloro.

- Definition of the pharmacokinetics of 11β in mice and preliminary formulation of 11β into liposomes. The liposomal form of 11β was found to have increased stability and prolonged circulation in blood. This result has prompted investigation of the pharmacokinetics and efficacy of liposomal 11β as an antitumor agent in mice.

## REPORTABLE OUTCOMES

**Manuscripts:**

John C. Marquis, Shawn M. Hillier, A. Nicole Dinaut, Denise Rodrigues, Kaushik Mitra, John M. Essigmann* and Robert G. Croy*, "A Bis-alkylating DNA Damaging Agent Tethered to a Ligand for the Androgen Receptor Reduces Skp2 Levels and Induces Apoptosis in Prostate Cancer Cells" Submitted February 2005.

**Patents applied for:**

### U.S. Provisional Application No. 60/552,322

TITLE: Methods and Compositions for Treating Cancer
DATE FILED: March 10, 2004
INVENTORS: Essigmann, J.M. and Croy, R.G.

DESCRIPTION OF THE INVENTION: The invention provides methods and compositions for treating cancer. Compounds of the invention can inhibit DNA repair pathways; induce apoptosis; and/or cause cell cycle arrest. Preferred compound of the invention are multifunctional agents that include i) a steroid receptor ligand domain, ii) a

nitrogen mustard domain (that can be inactivated) and iii) a linker that is resistant to hydrolysis under intracellular conditions. Compounds of the invention are useful in treating cancer. Compounds of the invention are useful for treating cancers that do not express or do not over-express a steroid receptor. Compounds of the invention are also useful in treating cancers resistant to DNA damaging compounds because of expression of anti-apoptotic factors (e.g. Bcl-2 and/or Bcl-xl expressing cancers or tumors) on activation of other survival mechanisms (e.g. mutation of PTEN), including mechanisms of apoptosis avoidance or apoptotic resistance. Compounds of the invention are particularly useful for treating prostate cancer that is refractory to treatment with conventional cytotoxic therapies as well as advanced metastatic disease that is resistant to hormonal antagonists.

**Degrees Supported:**

- Mr. Shawn Hillier has been supported on this grant. He is writing his dissertation and will graduate in June, 2005. Mr. Hillier was responsible for DNA repair, pharmacokinetic and antitumor studies reported in the application.
- Dr. Kaushik Mitra finished his postodoctoral work last year and is working in drug synthesis at Merck and Co. He was part of the synthetic chemistry team.

## CONCLUSIONS

We are now engaged in experiments to define the amounts of DNA damage produced by the 11β compound and the fates of this damage in target and non-target cells in culture and tumor xenografts. This information will help us establish the sequence of events that lead to apoptosis. The results we have obtained in animal models are especially significant. The ability of the 11β compound to prevent the growth of LNCaP cells in a mouse xenograft model provides evidence of the potential clinical activity of this compound. The activity of 11β will now be tested in additional xenograft models of human prostate cancer to assess the range of its antitumor properties.

Our research during the next year will focus on two goals: (1) identification of key events that lead to apoptosis in cells treated with 11β; and (2) optimizing antitumor responses to 11β in animal models of prostate cancer. We anticipate that identification of the signaling events originating from DNA adducts will provide clues to the reasons why the 11β compound is able to trigger apoptosis while other aniline mustard compouds such as chlorambucil do not. We now have sensitive methods to examine the formation of fate of 11β-DNA adducts in tumors. We will determine the levels of adducts in tumor and normal tissues that lead to antitumor responses and establish correlation with levels of DNA adducts that result in cytotoxic effects on cells in culture. We shall also proceed with the development of a more clinically useful formulation of 11β.

## REFERENCES

None included.

## APPENDICIES

None included.